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THE PROTEIN DENATURATION UNDER HIGH PRESSURE

Effects of pH and Some Substances on the Pressure Denaturation of Ovalbumin Solution.

By Chieko Suzuki, Keizo Suzuki, Kiyoshi Kitamura and Jiro Osugi

(*Received January 10, 1963*)

The effects of the pH and of the various added substances on the pressure denaturation of ovalbumin solution were examined by measuring the solubility as an index. The results are as follows: Ovalbumin has the maximum stability near pH 9 toward pressure-denaturation, the rate of pressure denaturation reaction of ovalbumin is proportional to the square root of hydrogen-ion concentration independently of the treated pressure and temperature, and the activation volumes are negative and the values depend on temperature but not on pH. Sulfate and glucose are inhibitors, and urea and ethyl alcohol are accelerators for pressure denaturation. And a little amount of calcium chloride and sodium chloride accelerates the pressure denaturation, but a large amount of them tend to inhibit it.

Introduction

We have studied the pressure denaturation of ovalbumin solution by measuring the solubility as an index[1,2]. It is well known that in general, the denaturation reaction is greatly affected by the pH of the protein solution and by the various added substances[3]. It interests us to examine how the pressure denaturation is dependent on the pH of the solution and how the substances, which affection the heat or urea denaturation, affect on the pressure denaturation.

Experimental

Material Ovalbumin was prepared by the method of Sørensen and Høyrup[4] and recrystalized three times. The solution was dialyzed for 24 hours against tap water. In the measurement of pH effect, the sample are adjusted with 0.1 *N* hydrochloric acid or 0.1 *N* sodium hydroxide, and in the measurement of the effects of some substances, the sample was compressed immediately after the addition of a substance to the protein solution indicated at pH 4.8 with 0.1 *M* acetate buffer.

High pressure apparatus and its manipulation The high pressure apparatus and its manipulation were the same as in the previous papers[1,5]. A sample solution charged in a poly-

1) K. Suzuki, *This Journal*, **28**, 24 (1958)
2) K. Suzuki, *This Journal*, **29**, 91 (1960)
3) F. W. Putnam, *The Proteins*, Edited by H. Neurath and K. Bailey, Academic Press, New York and London, Vol. I, P. 877 (1953)
4) S. P. Sørensen and Høyrup, *Compt. rend. trav. lab. Carlsberg*, **12**, 12 (1915)

vinylchloride sack was hydrostatically compressed at a constant pressure and temperature.

Assay of the extent of denaturation Assay of the extent of denaturation was carried out by measuring the solubilty as an index which was reported in the previous papers[1,5]: that is, the amount of soluble protein at pH 4.8 was colorimetrically measured at the wavelength of 550 mμ by the Biuret reaction.

Results and Discussion

Effect of pH In the range of pH where the acid or alkali denaturation can not occur, the effect of pH on the pressure denaturation of ovalbumin solution was studied. Fig. 1 shows that

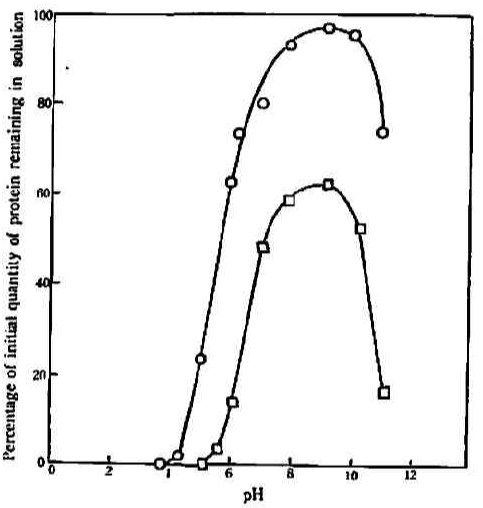

Fig. 1 Effect of pH on pressure denaturation. Samples were pressed for 5 minutes at 6500 kg/cm²: -○- and 7,500 kg/cm² -□- and 30°C.

the extent of denaturation by pressure greatly depends on pH of the solution, and ovalbumin has its maximum stability apparently* near pH 9 toward pressure denaturation at 6,500 and 7,500 kg/cm² and at 30°C. It is known that the maximum stability of ovalbumin is at pH 6.76 toward heat denaturation and near pH 8.0 toward urea denaturation[6]. Since the rate of pressure denaturation of ovalbumin is of first order[1,5], the rate constant k can be obtained by measuring the concentration of denatured protein molecule by compressing for a given time. The relation between the logarithm of the rate constant k and pH is shown in Figs. 2 and 3 (at 30°C and 25°C, respectively). To the experimental points the curves were fitted corresponding to the equation:

$$\log k = \log k_0 - n(\mathrm{pH}),$$

where k_0 and n are constants. That is, the linear relationships between $\log k$ and pH are satisfied. n's are the slopes of the lines and their values are 0.5 independent of pressure and temperature. It is shown that the rate of the pressure denaturation of ovalbumin is proportinal to the square

* The pH of protein solution should change under high pressure, since the weak electrolyte is more highly charged under pressure. The indicated values of pH are the values at atmospheric pressure.

5) C. Suzuki and K. Suzuki, *J. Biochem.*, **52**, 67 (1962)

6) R. B. Simpson and W. Kauzmann, *J. Am. Chem. Soc.* **75**, 5139 (1953)

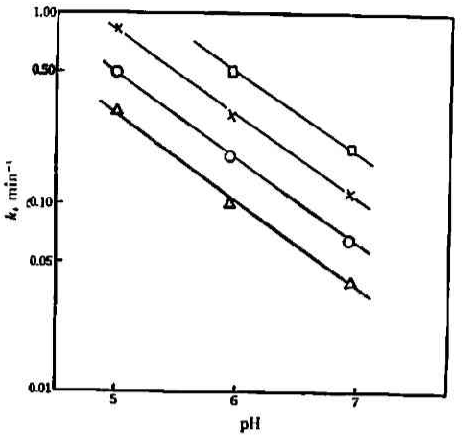

Fig. 2 Relation between logarithm of rate constant k and pH. Samples were pressed for 5 minutes at 7,500: -□-, 7,000: -×-, 6,500: -○- and 6,000 kg/cm²: -△-, and 30°C.

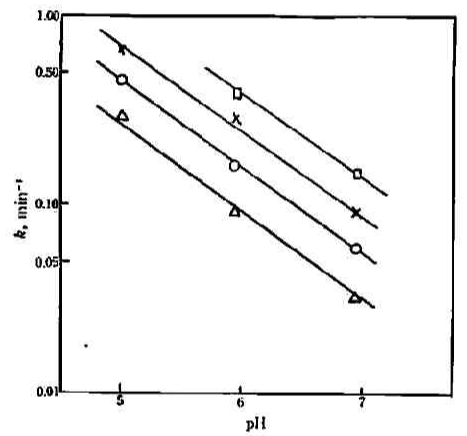

Fig. 3 Relation between logarithm of rate constant k and pH. Samples were pressed for 5 minutes at 7,500: -□-, 7,000: -×-, 6,500: -○- and 6,000 kg/cm²: -△-. and 25°C.

root of hydrogen-ion concentration.

Figs. 4 and 5 show the relations between the logarithm of the rate constant k and the applied pressure. Following the equation:

$$\partial \log k/\partial p = -\Delta V^*/RT$$

where ΔV^* is the activation volume, ΔV^* is -23.1 cc/mole at 30°C and -26.6 cc/mole at 25°C independently of pH. Therefore, the activation volume is more negative, the lower the temperature, in the pressure denaturation of ovalbumin. if pH of the sample was adjusted with sodium hydroxide. If the buffer solution is used instead of sodium hydroxide, the trend is similar but the absolute values are far larger as shown in the previous paper[1].

Effect of several electrolytes Fig. 6 shows the effect of several inorganic salts on the pressure denaturation. A little amount of sodium chloride and calcium chloride accelerates the pressure denaturation but a large amount of them tend to inhibit it. Sodium sulfate and ammonium sulfate are potent inhibitors. It is known that sulfates are the strong inhibitors in heat and urea denaturation, too[5~8]. On the contrary, a large amount of sulfate reduces solubilities of some proteins in water[9], and promote the precipitation reaction of denatured protein molecules*. And

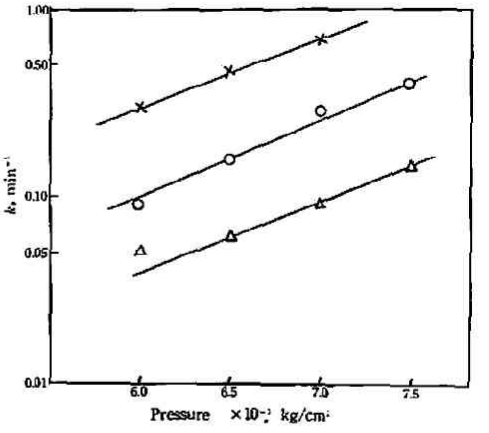

Fig. 4 Relation between logarithm of rate constant k and applied pressure. Samples were pressed for 5 minutes at pressures indicated at pH 5.0: - × -, 5.9: - ○ - and 6.9: - △ -, and at 30°C.

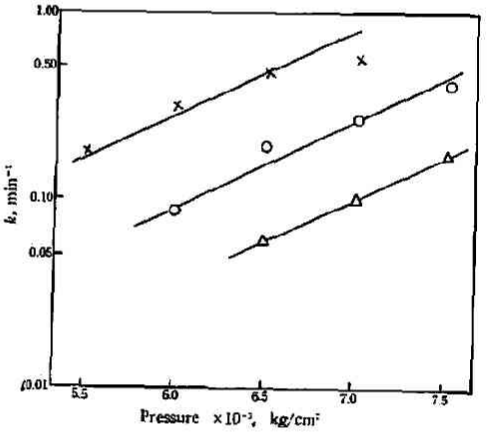

Fig. 5 Relation between logarithm of rate constant k and applied pressure. Samples were pressed for 5 minutes at pressures indicated at pH 5.0: - × -, 5.9: - ○ - and 6.9: - △ -, and at 25°C.

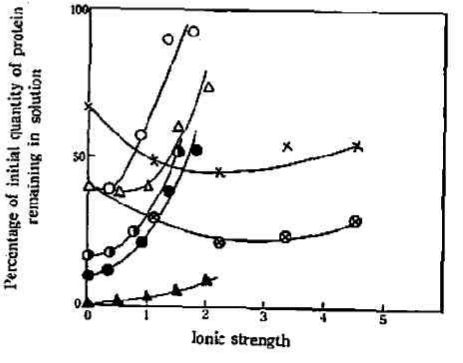

Fig. 6 Effects of electrolytes on pressure denaturation.
- ○ -: Na_2SO_4, at 5,000 kg/cm² and 25°C
- ● -: Na_2SO_4, at 5,500 〃
- △ -: NaCl, at 5,000 〃
- ▲ -: NaCl, at 6,000 〃
- × -: $CaCl_2$, at 4,500 〃
- ⊗ -: $CaCl_2$, at 5,000 〃
- ◑ -: $(NH_4)_2SO_4$, at 5,500 kg/cm² and 20°C

Other denaturation condition: pressure duration is 5 minutes, and pH is 4.8 with 0.1 M acetate buffer.

7) H. Chick and C. J. Martin, *J. Physiol.* (London), **40**, 404 (1910), **45**, 61 (1912)
8) A. Beilinson, *Biochem. Z.*, **213**, 399 (1929)
9) E. J. Cohn, *Chem. Revs.*, **19**, 241 (1936)

* We frequently experienced that sodium sulfate accelerated the precipitation of protein solution which has already denatured.

The Review of Physical Chemistry of Japan Vol. 32 No. 1 & 2 (1962)

also the gelation of ovalbumin by high pressure are accelerated by the addition of electrolytes (calcium chloride, sodium chloride and sodium sulfate)[10]. In general, the solubility of protein in water at the isoelectric point decreases by denaturation, that is, the conformation of the denatured protein molecules should favor the aggregation of them. We can not yet interpret such counter-effects of the electrolytes on the denaturation and on the aggregation of protein molecules.

The change of the extent of the denaturation with the concentration of acetate-buffer solution is shown in Table 1. It shows that the denaturation reaction of ovalbumin by pressure is accelerated with the increase of the acetate-buffer concentration.

Table 1 Effect of buffer concentration (pH 4.8 acetate buffer)

Buffer conc. (M)	C/Co
0	0.730
0.07	0.572
0.22	0.605
1.00	0.493
2.00	0.279

Samples were pressed for 5 min. at 5,000 kg/cm² and 20°C.

Effects of some organic substances Fig. 7 shows the effect of the low concentration of urea by which urea denaturation can not occur on pressure denaturation. It is found that urea is a strong accelerator for pressure denaturation, and the lower the pressure, the effect seems to be greater. Simpson and Kauzmann[6] have observed that the rate of the change of optical rotation of ovalbumin in a urea solution (urea conc. 6.25 *M*) at 0°C are remarkably accelerated by hydrostatic pressure of 610 kg/cm², and interpreted that it is connected with over-all decrease in volume by the urea denaturation. Their experiment shows that the activation volume of urea denaturation is negative. We found that the activation volume of pressure denaturation is negative, too. In view of these facts, it may be accepted that urea and pressure are the factors complementary for each other in the denaturation of ovalbumin solution.

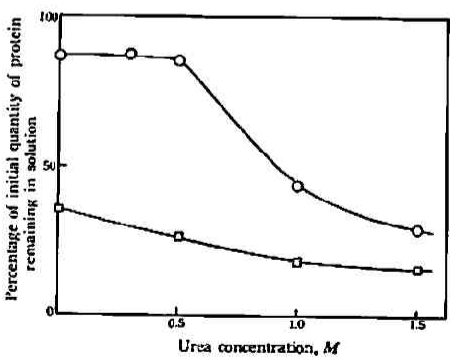

Fig. 7 Effect of urea on pressure denaturation. Samples were pressed for 5 minutes at 4,500: -○-, and 5,000 kg/cm²: -□-, and 25°C, indicated at pH 4.8 with 0.1 *M* acetate buffer.

The effects of ethyl alcohol and glucose on the pressure denaturation is shown in Figs. 8 and 9 respectively. They show that ethyl alcohol is a potent accelerator but glucose is a inhibitor for

10) C. Suzuki and K. Suzuki, *Arch. Biochem. Biophys.* In press.

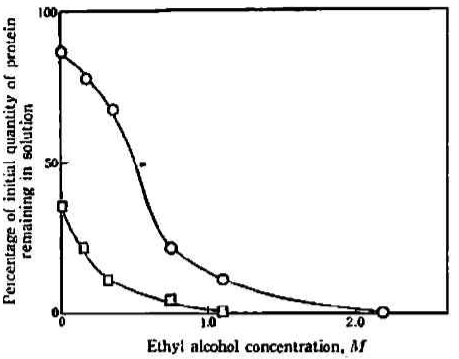

Fig. 8 Effect of ethyl alcohol on pressure denaturation. Samples were pressed for 5 minutes at 4,500: -○- and 5,000 kg/cm²: -□-, and 25°C indicated at pH 4.8 with 0.1 *M* acetate buffer.

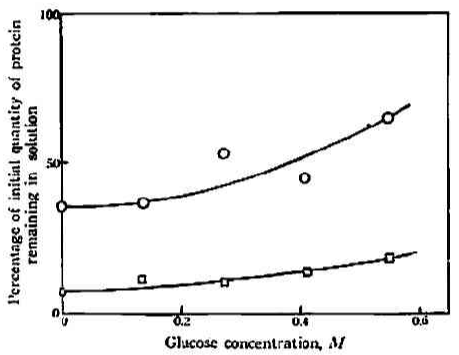

Fig. 9 Effect of glucose on pressure denaturation. Samples were pressed for 5 minutes at 5,500: -□- and 5,000 kg/cm²: -○-, and 25°C indicated at pH 4.8 with 0.1 *M* acetate buffer.

pressure denaturation as well as for urea denaturation[6,7].

In conclusion, within the extent of our investigation, the effects of pH and of the presence of electrolytes and organic substances on the pressure denaturation of ovalbumin is similar to the effects on the heat and urea denaturation.

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